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Stress Effects on Late Pregnancy in the Fluying-Fox, *Pteropus scapulatus*

W. Richard Dukelow[1], C. S. T. Pow, J. H. Kennedy and Len Martin

Department of Physiology and Pharmacology, University of Queensland, St. Lucia, Australia

ABSTRACT—Twenty one flying-foxes (*Pteropus scapulatus*) were alotted to three experimental groups. Group I received no handling until the final examination period. Group II was transported, anaesthetized, and blood sampled approximately 3 weeks before the final examination period. Group III received the same treatment as Group II but with the incorporation of a laparoscopic examination. High levels of abortion were noted in all groups (40.0, 20.0 and 45.5% for groups I, II and III respectively) during the three week experimental period but was not increased by the use of laparoscopy. Following the second period of examination stress, overall abortion rates for the total gestation period were 100, 80 and 90% for the three groups respectively, emphasizing the extreme stress-induced abortion susceptibility of this species. Nevertheless, the usefulness of laparoscopy to examine selected stages of the gestation was demonstrated. Over 54% of the animals diagnosed pregnant at the first laparoscopic examination were still pregnant at the subsequent examination 3 weeks later. Progesterone levels of 15.9 ng/ml or higher were found in all pregnant animals and were never above 4.3 ng/ml in nonpregnant animals.

INTRODUCTION

The Order Chiroptera (bats) has over 951 species distributed over 187 genera within 19 families [1]. These can be divided into the two sub-orders of Microchiroptera and Megachiroptera. The latter are found in the single family *Pteropodidae* consisting of 83 genera and 150 species. In Australia, they are represented by five genera and nine species. These flying foxes are characterized as large (up to one kg), frugivorous bats not possessing echolocation abilities, and as a nonhibernating species. They typically are found in the tropical forests where native fruits and flowers are abundant [2].

Pteropus scapulatus (little red flying fox) is the smallest of the Pteropid flying foxes found in Australia. This highly nomadic species is widespread throughout eastern Australia from arid inland areas to the coast. *P. scapulatus* mates in November and December (the Australian spring) and usually delivers a single offspring from the duplex uterus in April or early May (autumn) [3].

Few studies have been conducted on the reproductive physiology of flying foxes until recent years but successful captive breeding has been achieved [4, 5]. Some have suggested possible common neurological pathways between the megachiropterans and primates [6].

The present research was stimulated by analysis of data collected in this laboratory in 1984 after the capture of feral *P. scapulatus* in February with subsequent movement (in March) of 29 late pregnant animals. This movement stress resulted in the subsequent abortion of at least 86% of the pregnancies [7]. The objectives of the present studies were to a) determine if laparoscopy could be used to examine the conceptus in mid-pregnancy in *P. scapulatus* and b) determine the extent to which laparoscopy and associated stressors cause abortion in this species.

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[1] Present address and address for reprints: Endocrine Research Center, Michigan State University, East Lansing, MI 48824, U.S.A.

MATERIALS AND METHODS

Husbandry

Adult female *P. scapulatus* were caught wild at Monto, Queensland, Australia in March 1986 and held in open air cages 6×2×2 m with males throughout the breeding season. The animals were fed daily a chopped fruit (papaya, mango, grapes, pears, peaches, rock melons and honeydew melons) diet supplemented with calf and pig milk replacer 2–3 times each week and water *ad libitum.*

Experimental design

Twenty-one adult females were randomly allocated to one of three treatments as follows: Group I (negative control group) (five animals). These females were not initially restrained, examined or transferred. They remained in their colony cage with the males until March 14, 1989. Group II (positive control group) (five animals). These females were restrained and transported on February 20, 1989 from the University of Queensland Veterinary Research Farm to the departmental animal house, a distance of 12 km. These animals were then anaesthetized and a blood sample was taken as described below. Group III (laparoscopy group) (eleven animals). These females were restrained, transported, anaesthetized and blood sampled like the females in Group II. Additionally, each animal was examined laparoscopically.

Anaesthesia, blood sampling and blood analysis

Each animal in Groups II and III was anaesthetized with a mixture of 10 mg ketamine-HCl (Ketamine Injection®, Jurex Pty, Ltd., Riverstone, N.S.W.) and 2 mg xylazine (Rompun®, Bayer Australia Ltd., Botany, N.S.W.) i.m. in 0.2 ml volume. After anaesthesia, 0.5 ml of blood was drawn from the uropatagial vein [8] with a 25 ga, 19 mm needle attachted to a 1 ml tuberculin syringe. This sample was then placed in a 1 ml plastic vial containing 5 μl lithium heparin and gently mixed. The tubes were then placed in a refrigerator at 4°C until centrifuged the same day at 1500×*g* for 10–15 min. Plasma was separated and stored frozen in 1 ml plastic vials.

Progesterone analysis

The progesterone concentration in plasma was measured by radioimmunoassay using an antiserum to progesterone (prepared by Dr. R. I. Cox, Division of Animal Production, CSIRO, Prospect, N.S.W., Australia). ^{3}H-progesterone (2.04 TBq/mmol) was obtained from Amersham Australia.

A plasma pool containing progesterone was included in each assay to calculate inter- and intra-assay coefficients of variation. Parallelism was demonstrated using graded dilutions (1/20 to 1/1) of a plasma sample containing approximately 10 ng of progesterone per milliliter.

For assay, 50 μl plasma samples were diluted to 500 μl with 0.06 M phosphate-gelatin buffer (pH 7.2) and 50 μl aliquots in triplicate were extracted in 3 ml of freshly redistilled hexane (May and Baker Australia Pty Ltd.) and dried under nitrogen (average extraction efficiency 89.6%; n=3). Standards were prepared and stored in ethanol and various concentrations (0–2048 pg/tube). These were subsequently dried under nitrogen in triplicate for each assay. Extracts and standards were resuspended in 100 μl of buffer and allowed to stand at room temperature for three hr prior to the addition of antiserum at 1 : 8500 dilution in 100 μl of ^{3}H-progesterone (10,000 cpm) in the same buffer.

Tubes were incubated overnight at 4°C followed by separation of bound and free steroid with 300 μl of 0.625% charcoal-0.0625% dextran in buffer for 10 min at 4°C. Following centrifugation the supernatant was removed, added to 3 ml scintillation fluid (toluene, containing 0.3% PPO, 0.03% POPOP and 5% acetic acid) and radioactivity was measured in an LKB scintillation counter.

A standard curve was calculated according to a logit transformation and progesterone concentrations in unknown plasma samples were determined from this curve and expressed as nanograms of progesterone per milliliter of plasma. The sensitivity of the assay (caculated as two standard deviations from the blanks) was typically less than 16 pg/tube. The inter- and intra- assay coefficients of variation were 8.0% and 7.8% respectively.

Laparoscopy

The basic laparoscopic procedure was that described for small mammals [9] but modified for use in the flying fox based on preliminary laparoscopic examination in this laboratory [5, 7]. After clipping of the abdominal fur, the area was swabbed with a dilute solution of chlorhexidine gluconate (Hibitane®, ICI, Villawood, N.S.W.) in 20% ethanol. The animal was restrained in dorsal recumbency and the trocar-cannula inserted after initial incision through the skin, abdominal wall and peritoneum. The trocar was not forced through tissue to avoid the possibility of uterine puncture. The incision was approximately 5–10 mm posterior to the base of the sternum. A 4 mm paediatric laparoscope (Model 7200B, 30°, Karl Storz and Company, Tuttlingen, West Germany) was used. Insufflation was with nitrogen *via* the cannula gas attachment. A 19 ga 38 mm needle, attached to a 1 ml tuberculin syringe, was inserted through the lateral skin and body wall area to allow manipulation of the internal organs and, if necessary, to remove urine from the bladder to aid visibility. Since this needle is 1.08 mm in outer diameter, it was also used as a measuring device to approximate uterine size. During laparoscopy attempts were made to view the ovaries, oviducts and uterine horns of each animal, to ascertain the horn of pregnancy and, if no pregnancy was evident, to look for signs of abortion or resorption. In a preliminary laparoscopic examination of a female believed to be nonpregnant, the entire duplex reproductive tract was easily seen with a thickening and whitening of the cervical end of the right uterine horn and a yellow resorption site near the right tubal uterine junction where implantation normally occurs [10].

Laparoscopic examinations were completed in five min or less on each animal, the cannula-laparoscope removed and the abdominal wall and skin layers closed with 2–0 synthetic gut sutures. Of eleven animals receiving laparoscopic examinations, four subsequently (1–3 days) picked sutures free and were reanaesthetized and resutured with the addition of stainless steel Autoclips® (Clay-Adams, Parsippany, NJ, U.S.A.) placed on the skin. All initial laparoscopic examinations were completed on February 20–21, 1989.

After examination the animals were returned to cages in the animal house. All abortions were recorded.

On March 14, 1989 all animals in the three groups were brought again for pregnancy assessment by laparoscopic examination and/or palpation. All animals except those obviously pregnant by palpation were laparoscopically examined, including those known to have aborted. Again, a blood sample was collected for progesterone analysis.

Even though the basic research protocol ended with the March examinations, all pregnant animals were followed through to abortion or parturition to further assess the effect of late pregnancy anaesthesia, bleeding and laparoscopic stress.

RESULTS

The results of the initial laparoscopic examinations for pregnancy are shown in Table 1.

Based on subsequent examinations (and initial sample progesterone assays of the females in Group II) all of the 21 animals were pregnant at the start of the studies. In Group III, 5 of 11 pregnancies were in the right uterine horn. In Group I the two females that eventually aborted were pregnant in the left horn. Thus, where the side of pregnancy was definitely known, 5 of 13 (38.5%) were in the right horn. This difference was not statistically significant ($P>0.20$).

Of the 11 laparoscoped animals, 5 (45.5%) aborted after a single laparoscopic examination. These occurred 1, 4, 5, 7 and 16 days after laparoscopy. Aborted foetuses ranged from 7 to 20 gm. Progesterone values of pregnant animals ranged from 15.9 to 79.7 ng/ml. There was no significant relationship between progesterone level and subsequent abortion. In fact, the female with lowest progesterone level (number 12) maintained pregnancy through a second laparoscopic examination 32 days later. One animal that subsequently aborted (number 18) was not assayed for progesterone initially.

The results of the second laparoscopic examination are shown Table 2. In the negative control group (I) 3 of 5 animals were still pregnant where-

Table 1. Laparoscopic and endocrinological pregnancy analysis of flying foxes in Group III and outcome following initial laparoscopy (February) and examination 22 days later

Animal Number	Body Wt. (g)	Preg?	Side of Pregnan	Preg. Horn Diam. (mm)	Progesterone (ng/ml)	Outcome after initial laparoscopy
11	376	yes	left	14.0	72.8	Aborted, 1 day
12	306	yes	right	5.5	15.9	No effect
13	384	yes	left	11.0	79.7	No effect
14	292	yes	right	9.0	21.4	No effect
15	426	yes	right	17.0	25.4	No effect
16	272	yes	left	15.0	32.4	Aborted, 4 days
17	345	yes	right	15.0	62.7	No effect
18	293	yes	left	16.0	—	Aborted, 7 days
19	402	yes	left	12.5	26.4	Aborted, 16 days
20	406	yes	right	10.0	47.5	No effect
21	340	yes	left	9.0	66.4	Aborted, 5 days

Table 2. Laparoscopic and endocrinological pregnancy analysis of flying foxes in all groups at final laparoscopic examination

Animal Number	Body Wt. (g)	Pregnancy Octcome	Progesterone (ng/ml)	Abortion Data	Foetal Wt(g)
Group I					
1	312	Pregnant	39.2		
2	309	Aborted	1.5	unknown	unknown
3	396	Pregnant	101.1		
4	316	Aborted	3.4	unknown	unknown
5	312	Pregnant	98.2		
Group II					
6	262	Pregnant	60.2		
7	540	Pregnant	97.3		
8	—	Aborted		March 10	19.0
9	244	Pregnant	67.1		
10	283	Pregnant	63.3		
Group III					
11	329	Aborted	2.2	Feb. 21	12.0
12	300	Pregnant	34.9		
13	386	Pregnant	53.0		
14	306	Pregnant	36.8		
15	422	Pregnant	40.0		
16	—	Aborted		Feb. 24	10.0
17	350	Pregnant	1.5		
18	273	Aborted	4.3	Feb. 27	20.0
19	264	Aborted	3.0	March 9	8.0
20	357	Pregnant	103.1		
21	325	Aborted	2.9	Feb. 26	7.0

as the other two showed evidence of abortion (both in the left horn). The pregnant animals were obviously pregnant (subsequently confirmed by progesterone levels) and were not subjected to laparoscopic examinaiton. Of the four remaining animals in Group II (handled and bled but not laparoscoped in February) all were obviously pregnant (confirmed by progesterone levels). The fifth animal had been pregnant and aborted a 19 gm foetus on March 10th. Of the 11 animals in Group III (see above) six were pregnant at the time of the final examination. All but one exhibited progesterone levels indicative of pregnancy and ranging from 34.9 to 103.1 ng/ml. The exception is animal 17 who had a level of 1.5 ng/ml progesterone. This animal aborted a 3.3 gm foetus the next day. This female had a 15 mm diameter pregnant horn at the initial laparoscopy (Table 1). Comparison of foetal sizes with females numbered 11, 16 and 18 (who had uterine horn diameters of 14–16 mm and subsequently aborted within 7 days of laparoscopy) indicates this foetus should be between 10 and 20 grams. We thus conclude that the foetus of animal 17 had died but was not aborted until the day after the second laparoscopic examination. Abortion rates between Groups I, II and III were analyzed by factorial Chi-squared tests for factors and interaction. Laparoscopy Group III had no significant effect ($P<0.10$) on abortion rate. Although animal numbers were low in Groups I and II, abortion rate was significantly higher ($P<.005$) in Group I.

All animals were returned to large outdoor cages on March 18, 1989 and animals not delivering by June 15 were considered to have aborted and the foetus lost. A final analysis of Group I showed that all animals had been pregnant but subsequently aborted. None of these animals were ever laparoscoped while pregnant. In Group II, two live births occurred on April 23 and May 3 (females 7 and 6 respectively). Neither animal had ever been laparoscoped. In Group III one live birth occurred, on May 10, to female 15. This animal had been laparoscopically examined once in the initial (February) examination. Interestingly, this female had the largest uterine horn diameter in Group III (Table 1) and thus may have been the earliest pregnancy in the group. This was the largest animal both at the initial (February) and subsequently (March) examinations in Group III. Only one other pregnant female (number 7 in Group II) wsa larger at the March examination and this female also delivered a live young, on April 23rd.

DISCUSSION

The high fertility rate of *P. scapulatus* obtained in captivity is reflected by the 100% overall pregnancy rate at the start of these studies, estimated conservatively at one-third of the gestation period. This is in accord with the report of Towers [7] who reported capture of a large number of *P. scapulatus* in Queensland in February 1984, all of which were pregnant. Similarly, all 42 mature *P. poliocephalus* (the grey headed flying fox) collected between April and September (normal time for gestation in this species) were pregnant.

In the present studies pregnancy occurred on both sides of the reproductive tract, apparently randomly (38.5% on the right). Others [10, 11] attributed alternation of sides with successive ovulations to *P. giganteus* but the evidence for this was minimal and cannot be confirmed from the present experiments. Towers [7] reported 9 of 17 (52.9%) *P. scapulatus* pregnancies were in the right uterine horn. There is evidence for a local endometrial reaction to the developing corpus luteum in a number of Chiropteran species and Bonilla and Rasweiler [12] proposed a countercurrent exchange of ovarian hormones between the arterial supply of the uterus and the ovarian venous or lymphatic drainage. Pow and Martin [13] have demonstrated evidence for such an exchange in *P. scapulatus*.

In the present studies all implantations appeared to occur cranially, near the tubal-uterine junction, in accord with the report by Marshall [10].

The laparoscope proved to be a simple and useful tool to examine the reproductive tract, although the cumulative effect of capture, transport, anaesthesia, bleeding and laparoscopy obviously resulted in abortion. However the rate was not significantly higher than in control (Group I) animals, in fact a significantly greater percentage of the control animals aborted than those in Group

II. The present studies do confirm the high susceptibility of these animals to abortion [7]. It is not evident that laparoscopy caused this stress. This is not to suggest that abortion cannot occur due to other natural causes. Since the animals were held in captivity for almost three years, age may have been a factor for some females. Nelson [4] reported that small, all-female groups were formed after conception. Thus the continued presence of breeding males in the present trials may have exerted a negative effect. Two of the negative control animals aborted before any of the group were transported and moved. The remaining three animals aborted subsequently without laparoscopic examination. In Group II, three of five animals aborted without laparoscopic examination. Thus eight of ten animals (80%) aborted, six of which received handling, anaesthesia and bleeding stress. With animals which were also subjected to laparoscopy, nine of ten (90%) subsequently aborted. Of these nine, seven had known dates of abortion and, of these, only four were within seven days of the laparoscopy examination. Two of these animals were laparoscopically examined twice with abortion after the second examination. One animal, laparoscopically examined on February 20, and subjected to anaesthesia, palpation and blood sampling on March 14, subsequently gave birth on May 10th. Thus there appears to be wide variations in the susceptibility to these stressors, but with a substantial level of abortion.

Progesterone levels were an accurate measure of pregnancy. Towers [7] reported mean nonpregnant progesterone levels in *P. scapulatus* to be 4.0 $\pm$0.5 ng/ml and never over 10 ng/ml. He reported February pregnancy levels to range from 16 to 135 ng/ml. May pregnancy levels ranged from 102 to 195 ng/ml. In the present study, nonpregnant animals averaged 2.9 ng/ml of progesterone (ranges 1.5 to 4.3). February pregnant animals averaged 45.1 ng/ml and ranged from 15.9 to 79.7 ng/ml. March pregnant animals averaged 66.2 ng/ml and ranged from 34.9 to 103.1 ng/ml. No pregnant animal was found with a progesterone level of less than 15.9 ng/ml.

It is significant that of eleven animals initially subjected to complete stress (Group III) six remained pregnant for the subsequent three weeks indicating, that while the abortion rate is high, limited observations can be carried out to study mid-pregnancy development within the uterus. The effect of handling and laparoscopic stress during very early pregnancy was not examined in the present study.

The high incidence of abortion in Groups I and II confirm the susceptibility of *P. scapulatus* to stress-induced abortion and emphasizes the need for compassionate and careful handling of the animals in captivity.

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